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Production of α-keto acids: 2. Immobilized whole cells of *Providencia* sp. PCM 1298 containing L-amino acid oxidase

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A number of bacteria belonging to the genera Proteus, Providencia, Pseudomonas *and* Erwinia *have been tested for their capacity to oxidize* L*-amino acids to their corresponding α-keto acids. Members of the* Proteus *and the* Providencia *genera were active towards various* L*-amino acids. Immobilized cell preparations of* Providencia *sp. PCM 1298 were shown to form up to 80 mg α-keto-γ-methiol butyric acid from* L*-methionine per g of gel preparation (containing 4% w/w cells) per day. The productivity was highly dependent on the size of the beads. Oxygen appeared to be the rate-limiting substrate and oxygen transfer rates of 3–4 μmol cm^{-2} h^{-1} were calculated. The entrapment of activated charcoal to remove H_2O_2 formed during the oxidation extended the half-life of the immobilized biocatalyst considerably. A decrease in* L*-amino acid oxidase [*L*-amino acid: oxygen oxidoreductase (deaminating); EC 1.4.3.2] activity during operation could be compensated for by reinoculation of the alginate-entrapped cells in fresh growth medium, allowing use of these preparations of immobilized bacterial cells for more than one month.*

Keywords: Hydrogen peroxide; L-amino acid oxidase; *Providencia* sp.; immobilized cells; α-keto acid production; activation *in situ*

Introduction

Recently, there has been considerable interest in the potential use of α-keto acids for the treatment of chronic uraemia. In this context, the keto analogues of the essential amino acids are of particular interest. The chemical synthesis of some of these analogues is not possible on a commercial basis. A promising alternative method for the production of α-keto acids is the utilization of amino acid oxidases [L-amino acid: oxygen oxidoreductase (deaminating); EC 1.4.3.2 and D-amino acid: oxygen oxidoreductase (deaminating); EC 1.4.3.3], which transform amino acids to the corresponding keto acids. The cost of commercially available oxidases (from hog kidney and snake venom) is high and therefore we have been involved in finding a suitable microbial source.

In previous studies on the development of a process for the production of α-keto acids from the corresponding amino acids, a D-amino acid oxidase present in immobilized whole cells of *Trigonopsis variabilis* was studied.[1] One of the advantages of employing an enzyme specific for the D-isomer of the substrate in a process would be the simultaneous production of optically active L-amino acid together with the α-keto acid from racemic mixtures of the substrate. During recent years the price of L-amino acids has, however, decreased considerably and today most L-amino acids are less expensive than the corresponding DL-amino acids. We have therefore extended our investigations to the possible utilization of a microbial L-amino acid oxidase for the production of α-keto acids. To this end, we have studied immobilized bacteria containing L-amino acid oxidase. In this paper we describe some basic characteristics of immobilized viable cells of *Providencia* sp. PCM 1298.

Materials and methods

Chemicals

Alginate (Manucol DH batch no. 430041 E6896) was obtained from Alginate Industries (Girvan, UK). Methionine and other chemicals were of analytical grade and were purchased from various commercial sources.

Culture conditions

For selection, the bacteria were grown on a medium containing bactopeptone (1%), casein hydrolysate (0.2%), yeast extract (0.2%) and NaCl (0.6%), pH 7.2–7.4. The microorganisms were transferred from agar slants to 25 ml medium and incubated for 20 h at 30°C with shaking (50 rev/min).

The selected strain (*Providencia* sp. PCM 1298) was cultivated for further studies on the above medium supplemented with 10 mM $CaCl_2$ instead of NaCl for 14 h. The cells were harvested by centrifugation (15 min, 10000*g*)

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and washed with 50 mM Tris–HCl buffer, pH 7.5. The average yield was 16 g cell (wet weight) per litre of medium.

Immobilization of Providencia *sp. PCM 1298 cells*

Immobilization of *Providencia* cells by entrapment in Ca^{2+}-alginate gels was performed according to the following standard procedure. Wet cells (400 mg) suspended in H_2O (1.1 ml) were mixed with sodium alginate (8.5 g) (5%, autoclaved for 20 min) and the mixture was subsequently added dropwise into 50 mM $CaCl_2$. After 30 min, the beads were collected by filtration and washed with 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM $CaCl_2$.

When activated charcoal (2 h at 120°C) or manganese oxide was coimmobilized with the cells it was done by adding this agent (250 mg) to the alginate solution, which was subsequently sonicated in order to disrupt larger particles of the agent. The cells (400 mg) suspended in H_2O (0.85 ml) were then added and the immobilization was carried out as described above.

Cultivation of immobilized cells

The beads (3 g wet weight) with cells of *Providencia* were cultivated in the standard medium (25 ml) on a shaker at 30°C for 14–16 h. The gel beads were then filtered off and washed extensively with 10 mM $CaCl_2$ followed by 50 mM Tris–HCl buffer, pH 7.5. The washed beads were assayed for L-amino acid oxidase activity. 'Activation' of used immobilized cells was done using this same procedure.

Solubilization of alginate gel

Gel particles (1 g) containing *Providencia* cells were solubilized by suspending them in 0.1 M sodium phosphate buffer (5 ml), pH 7.5. After shaking for 2 h at room temperature the beads were completely solubilized. After centrifugation (10 min, 10 000 *g*) the cells were assayed for L-amino acid oxidase activity.

Enzyme assays

The L-amino acid oxidase activity within whole cells of *Providencia* was assayed either by a colorimetric method using dinitrophenylhydrazine or by reverse-phase h.p.l.c. as described previously.[1]

Catalase activity was determined spectrophotometrically at 240 nm.[2]

Results and discussion

Selection of L-amino acid oxidase producer

Some strains belonging to the genera *Proteus*, *Providencia*, *Pseudomonas* and *Erwinia* were examined for L-amino acid oxidase activity. Two strains of *Providencia*, six strains of *Proteus* and none of *Pseudomonas* contained L-amino acid oxidase, as listed in *Table 1*. In addition to the oxidase, these strains also produced catalase.

Among the examined strains the highest activity was obtained in cells of *Providencia* sp. PCM 1298 (*Table 1*), which was selected for further studies. Maximum L-amino acid oxidase activity was observed in the late logarithmic phase of bacterial growth, i.e. 13–14 h after inoculation, as shown in *Figure 1*. At this point, ~4 μmol keto acid is produced per mg of cells (wet weight) per hour. This is considerably higher than the productivity obtained with the D-amino acid oxidase in *T. variabilis* cells.[1] Furthermore, it was found that the initial catalase activity was ~30 times higher than the oxidase activity on a stoichiometric basis.

Table 1 L-Amino acid oxidase activity within whole cells of various bacterial strains. L-Methionine, 10 mM in 50 mM Tris–HCl buffer, pH 7.5, was used as substrate

Strain	Activity (μmol keto acid mg^{-1} wet cells h^{-1})
Proteus mirabilis[a]	0.43
mirabilis D-42 Macana[a]	0.54
mirabilis PCM 1353[b]	1.10
vulgaris[a]	0.39
vulgaris TM[c]	0.62
vulgaris PCM 1350[b]	1.10
rettgeri PCM 1383[b]	0
morganii PCM 1371[b]	0
Providencia sp. PCM 1270[b]	0.45
PCM 1274[b]	0
PCM 1297[b]	0
PCM 1298[b]	1.90
Pseudomonas aeruginosa PCM 1109[b]	0
fluorescens PCM 2123[b]	0
putida PCM 2124[b]	0
sacharophila PCM 2119[b]	0
Erwinia carotowora PCM 2056[b]	0

[a] From the Department of Microbiology, University of Lund
[b] PCM, Polish Collection of Microorganisms, Institute of Immunology and Experimental Therapy, Wroclaw
[c] From the Department of Technical Microbiology, University of Lund

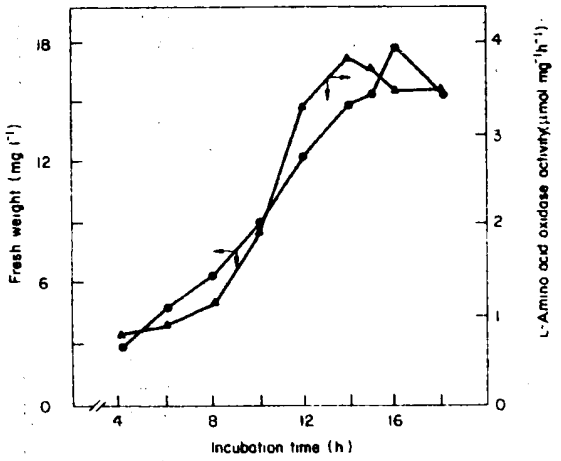

Figure 1 Cultivation of *Providencia* sp. PCM 1298 on a complex medium at 30°C

Table 2 shows that a number of L-amino acids can be converted to the corresponding α-keto acids with relatively high activities. The essential amino acids are of special interest, and most can be converted to the corresponding keto acids. Methionine was selected as a representative substrate and was used in subsequent studies. Since no difference in activity could be observed when L- or DL-methionine was used (*Table 2*) the racemic substrate was used in most experiments.

When whole cells of the bacteria are used for the oxidation of methionine (and other amino acids) to the corresponding keto acid no side reactions are observed and consequently no isolation of the enzyme was attempted.

Immobilization of Providencia *cells*

Whole cells of *Providencia* were immobilized by entrapment in Ca^{2+}-alginate. This immobilization method is simple and inexpensive and the cells remain viable after immobilization. Some basic characteristics of this immobilized biocatalyst were studied, as outlined below.

Table 2 Relative activity of L-amino acid oxidase within whole cells of *Providencia* sp. PCM 1298 towards various substrates

Substrate[a]	Relative activity
L-Methionine	100
D-Methionine	0
DL-Methionine	100
DL-Ethionine	105
DL-Alanine	0
DL-Valine	0
DL-Norvaline	70
DL-Leucine	200
DL-Norleucine	230
DL-Isoleucine	0
DL-Aspartic acid	0
DL-Tyrosine[b]	60
DL-Phenylalanine	180
DL-Tryptophan	80
DL-Lysine	0
DL-Arginine	0
DL-Histidine	0
DL-Threonine	0
DL-Proline	0
DL-Serine	0

[a] 5 mM D- or L-amino acid or 10 mM DL-amino acid
[b] Determined at lower substrate concentration due to poor solubility

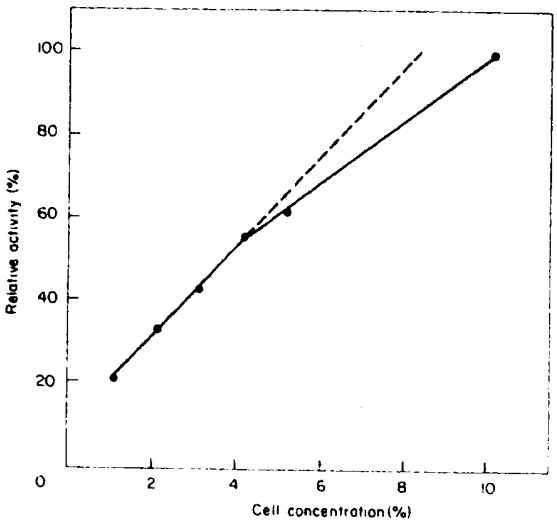

Figure 2 Effect of cell content of the beads on the conversion of L-methionine to α-keto-γ-methiol butyric acid. The beads (φ 3.5 mm, 5.0 g wet weight) were packed in the reactor (16 x 100 mm) and the substrate (20 mM DL-methionine in 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM $CaCl_2$) was pumped through the reactor (20 ml h^{-1}) at room temperature and air was passed through the reactor (20 ml min^{-1})

Cell content of beads

A linear increase in productivity was observed with increasing cell content of the heads at low cell densities (<5% w/w), as shown in *Figure 2*. At higher cell densities, the productivity is no longer proportional to cell content, most likely due to diffusional barriers for the substrates. A similar relationship was observed in a corresponding system containing *T. variabilis* cells.[1] The rate-limiting substrate is probably oxygen, which has to be transferred from the gaseous phase to the immobilized biocatalyst. For efficient oxygen transfer an appropriate reactor design is required, and in our studies we utilized a 'trickle-bed' reactor.[1]

Size of beads

The size of the beads is another factor which strongly influences the apparent activity. *Figure 3* shows the productivity within a 'trickle-bed' reactor as a function of bead size. An increase in bead diameter from 2.4 to 3.5 mm reduces the expressed activity to ~35%. For comparison, the productivities obtained with immobilized cells of *T. variabilis* are also included.[1] Even though different cell concentrations have been used in the two studies, the productivities obtained are comparable. A possible explanation is a relatively diffusion-limited conversion. The rate-limiting substrate is most likely oxygen. An oxygen transfer rate of 3–4 μmol O_2 cm^{-2} h^{-1} can be calculated for all these reactors. Increased oxygen transfer should result in higher productivity. Under the conditions used, only the cells in the outer layer of the beads are effective in the catalysis and therefore the productivity is proportional to the effective surface area. Clearly, very small beads would result in a relatively high productivity. Good flow properties within the reactor, however, may be difficult to maintain with very small beads. A compromise between expressed activity and bead size has to be found for a large-scale process. Furthermore, other reactor designs should be considered for a higher oxygen transfer rate. With beads of diameter 2.4 mm a productivity of 80 mg α-keto-γ-methiol butyric acid per gram of beads (containing 4% cells w/w) per day can be achieved under the conditions used.

Flow rate

The conversion of amino acid to keto acid is highly dependent on the flow rate through the reactor, as shown in *Figure 4*. At a low flow rate, a quantitative conversion can be obtained which simplifies the isolation of the products when the L-amino acid is used as substrate. At

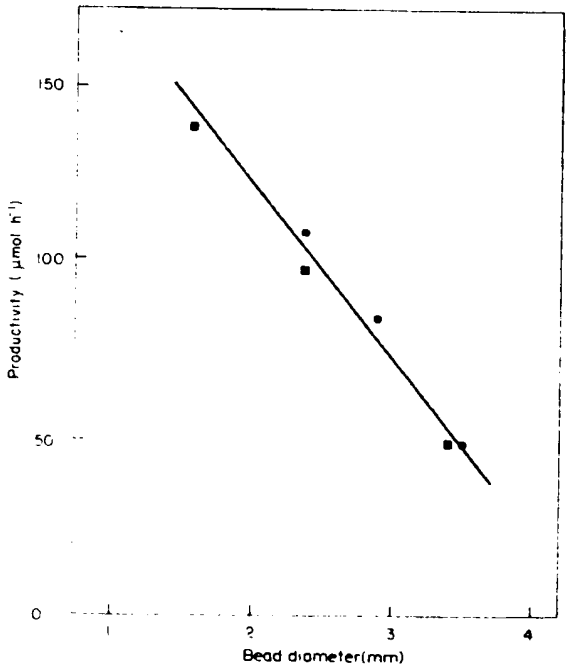

Figure 3 Productivity of α-keto-γ-methiol butyric acid from L-methionine as a function of bead size. ●, Immobilized cells of *Providencia* sp. PCM 1298; conditions as described in legend to *Figure 2*. ■, Immobilized cells of *T. variabilis*; data from ref 1

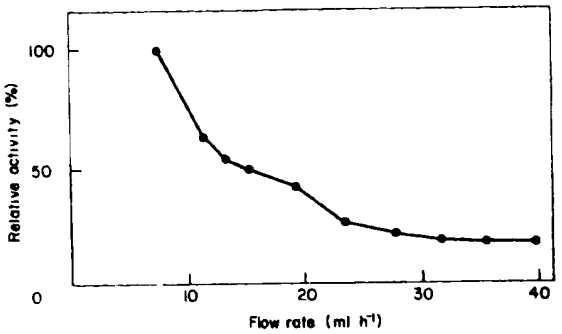

Figure 4 Effect of flow rate on the conversion of L-methionine to α-keto-γ-methiol butyric acid. Conditions as described in legend to *Figure 2*

higher flow rates the productivity is higher but an efficient separation of substrate and product is required. *Figure 4* shows that no further decrease in conversion is observed at relatively high flow rates. The reason for this constant conversion at higher flow rates is not fully understood. A similar behaviour was observed with immobilized cells of *Trigonopsis variabilis*.[1]

Operational stability

One of the most important features of immobilized biocatalysts for use in technical processes is the stability, in particular the operational stability. As pointed out earlier,[1] the removal of hydrogen peroxide formed in the oxidation is very important for the stability of the biocatalyst. This product can cause denaturation of proteins and can also react with α-keto acids to form the corresponding decarboxylated compound. Catalase [hydrogen peroxide: hydrogen peroxide oxidoreductase; EC 1.11.1.6] is a natural catalyst which efficiently degrades the harmful peroxide. Cells of *Providencia* contain relatively high amounts of catalase and the cells, therefore, appear to be very suitable for the oxidation. The enzyme is, however, denaturated by its own substrate, i.e. hydrogen peroxide, which may limit the applicability of this intrinsic catalyst.

The operational stability of immobilized *Providencia* cells was tested by continuously pumping a substrate solution through the 'trickle-bed' reactor. After an initial high, constant conversion for about two days a rapid decline in activity is observed, as can be seen in *Figure 5*. No increase in activity was achieved on repacking of the column, indicating that the decrease in activity was not due to changed flow properties of the reactor, e.g. channelling. Addition of FAD (0.1 mM) to the substrate stream did not change the activity and the decrease in conversion was apparently not related to loss of the flavin coenzyme, as has been reported for immobilized purified amino acid oxidase.[3] The initial stability can be explained by the presence of active catalase within the immobilized cells. The enzyme is, however, continuously inactivated by the hydrogen peroxide formed (addition of soluble catalase to the substrate stream increased the conversion rate significantly). The half-life of the amino acid oxidase is ~5 days, which is not sufficient for a commercial process. The amino acid oxidase seems to be less sensitive to hydrogen peroxide than the catalase.

If the accumulated hydrogen peroxide could be efficiently degraded an increased stability could be expected. A convenient way to achieve this is to coimmobilize an additional catalyst with the bacterial cells. In our studies on D-amino acid oxidase within immobilized cells of *T. variabilis* manganese oxide was used.[1] When the cells and the oxide particles were coentrapped in Ca^{2+}-alginate an efficient degradation of the hydrogen peroxide formed was obtained. The hydrolysis of the peroxide also leads to recycling of oxygen, which favourably influences the amino acid oxidase activity within the gel. In addition to manganese oxide we have also coimmobilized activated charcoal with the *Providencia* cells. The latter agent gave a somewhat longer half-life for the biocatalyst. The improved stability may be due to the fact that the charcoal particles are smaller in size than the oxide particles and, therefore, a more efficient interaction between the bacterial cells and the peroxide-degrading agent is achieved. It was observed that the activated charcoal initially absorbed some of the keto acid produced and ~0.14 mmol of α-keto-γ-methiol butyric acid was required for saturation of 1 g charcoal. At saturation, no decrease in peroxide-destroying capacity could be detected. The slight increase in conversion during the first two days of operation observed for the charcoal-containing catalysts (see *Figure 5*) can be explained by the fact that it takes some time before the charcoal is saturated with keto acid. Furthermore, it was calculated that 50 μg charcoal can efficiently remove the hydrogen peroxide formed by the L-amino acid oxidase activity of 1 g wet cells. As can be seen in *Figure 5*, the apparent half-life of the biocatalyst was extended to at least 11 days and the risk of a secondary reaction between the keto acid and the hydrogen peroxide (i.e. decarboxylation of the former) was reduced.

The operational stability was also influenced by the pH of the substrate solution passed through the reactor. The pH optimum for the amino acid oxidase within immobilized *Providencia* cells was determined to be 8.2. When charcoal was included in the gel a shift of pH-optimum appeared to take place, as can be concluded from *Figure 5*. When no charcoal was present in the beads (open symbols) a higher conversion was observed at pH 8.2 than at pH 7.4.

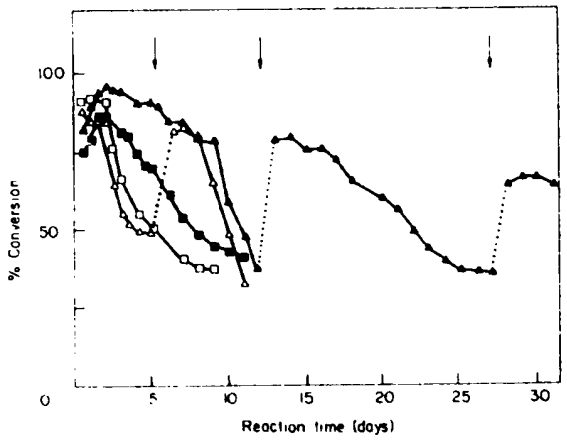

Figure 5 Continuous transformation of L-methionine to α-keto-γ-methiol butyric acid by immobilized *Providencia* cells. The beads (ϕ 2.4 mm, 10 g wet weight containing 4% cells (w/w)) were packed in the reactor (1.6 x 100 mm) and the substrate (20 mM DL-methionine in 50 mM Tris–HCl buffer containing 10 mM $CaCl_2$) was pumped through the reactor (8 ml h^{-1}) and air was passed through the reactor (20 ml min^{-1}). △, ▲, pH 7.4; □, ■, pH 8.2. Full symbols indicate coimmobilization of activated charcoal (2.5% w/w) with the *Providencia* cells. The arrows indicate 'activation' of the beads as described in Materials and methods

When charcoal was present in the beads (full symbols) the situation was reversed, i.e. higher conversion at pH 7.4 than at pH 8.2. Furthermore, it can be seen that the operational stability is considerably higher at pH 7.4 with the peroxide-degrading agent present in the beads.

Activation

The procedure to restore or increase the enzymatic activity of beads containing viable microbial cells by incubation in a nutrient medium has been described.[4-6] These studies discussed whether the increased activity was due to cell growth or to preferential formation of the enzyme in question. We have in a similar way incubated alginate-entrapped *Providencia* cells by placing the beads in the nutrient medium normally used for cell cultivation. As can be seen in *Figure 6a*, the increase in enzymatic activity with time is similar to that observed for cells in suspension (cf. *Figure 1*). The highest increase in activity was obtained at the lowest polymer concentration (2.5% w/w alginate). In this case, however, the number of cells increased to such an extent that the beads started to burst, releasing cells into the incubation medium. The somewhat lower activation observed at higher alginate concentrations (5 and 7% w/w) may be due to diffusional limitations and/or steric hindrance for extensive cell growth by these stronger gels. It can be concluded that the activation is at least partly due to an increase in the number of cells within the alginate beads.

When the incubated beads are dissolved (phosphate buffer) and subsequently assayed for amino acid oxidase, a similar activation pattern is observed, as shown in *Figure 6b*. A considerably higher total activity (10–15 times) is measured in this case since the diffusional barriers to oxygen discussed above are eliminated as the gel is dissolved. Clearly, only ~10% of the enzymatic activity within the beads is expressed due to diffusional limitations under the conditions used.

Activation of the immobilized biocatalyst is a powerful tool to prolong the operational life of the catalyst. Two fundamentally different approaches exist to do this. The first is based on a continuous slow growth of the immobilized cells by inclusion of a limited-growth medium in the substrate stream. This approach, however, requires extensive optimization studies to achieve an appropriate steady-state growth and it entails increased problems in the isolation of the product. The other approach, which appears to be simpler and more applicable, involves intermittent exposure of the immobilized cell to a complete growth medium for some time to allow restoration of the catalytic activity.

We have tested the latter method to restore the oxidase activity after continuous utilization of the catalyst for some time. From *Figure 5* it can be seen that activation is possible and that the activity is restored to some extent. Activation for a longer time might give complete restoration of oxidase activity. The presence of charcoal in the gel does not interfere with the activation. Furthermore, as shown in *Figure 5*, it is possible to activate the same catalyst several times and this will increase the applicability of this immobilized biocatalyst for the large-scale production of α-keto acids from the corresponding L-amino acids. This method of activation can extend considerably the operational life of the catalyst. In the previous study on D-amino acid oxidase within immobilized *T. variabilis* cells no activation was possible since the cells were not viable due to freezing, which was required to eliminate the transport barriers across the cell membrane for several amino acids.

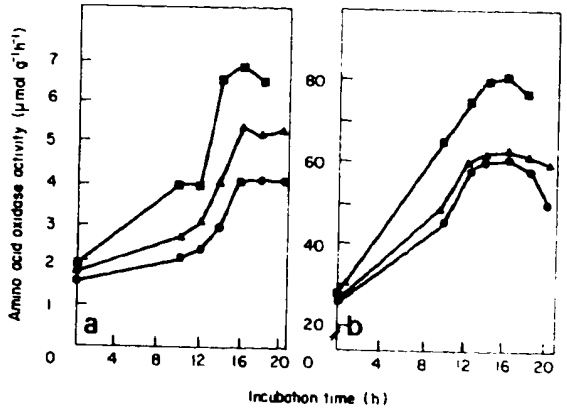

Figure 6 L-Amino acid oxidase activity of immobilized cells of *Providencia* after cultivation of the beads in a nutrient medium as described in Materials and methods. The cells were coimmobilized with charcoal (2.5% w/w). The initial concentration of cells was 2% (w/w). The cells were entrapped in: ■, 2.5% (w/w) alginate; ▲, 5% (w/w) alginate; ●, 7% (w/w) alginate; (a) intact beads; (b) solubilized beads

Conclusions

The data presented here should be a platform for the development of a commercial process for the production of α-keto acids from the corresponding L-amino acids. The major advantages of this immobilized biocatalyst are, in principle, the same as those for immobilized cells of *T. variabilis* containing D-amino acid oxidase,[1] i.e.

(1) a microbial source for the enzyme;
(2) a relatively broad substrate specificity of the enzyme;
(3) whole cells can be used without side reactions; and
(4) inexpensive and stable hydrogen peroxide-degrading agent (charcoal) can be used.

The immobilized *Providencia* cells, however, have some additional advantages which makes the system described in this paper even more attractive for large-scale operation. The enzyme is specific for the L-amino acid, which today is the most inexpensive substrate and the immobilized cells are viable, allowing activation of the biocatalyst when the activity has decreased to a low value.

Acknowledgements

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